Hyphal length in the forest floor and soil of subtropical, temperate, and subalpine forests

Takashi Osono

T. Osono
Center for Ecological Research, Kyoto University, Otsu, Shiga 520-2113 Japan
e-mail: tosono@ecology.kyoto-u.ac.jp

Abstract Fungi are a major component of soil biota in terrestrial ecosystems of various climatic regions, but few studies have compared the effects of soil layers and seasons on their abundance between sites with different humus forms and climatic conditions. In the present study, the hyphal length in the forest floor and soil was investigated in subtropical (ST), cool temperate (CT), and subalpine (SA) forests in Japan with an agar film method. The primary aim was to describe the seasonal variations in hyphal length in different layers of mull and moder humus forms in forests of these three climatic regions. The total hyphal length was generally higher in CT than in ST and SA and decreased with the soil depth. Seasonal changes in total hyphal length were observed in ST and in the lower slope of CT where mull humus developed. The length of darkly pigmented hyphae and its proportion relative to the total hyphal length were higher at the upper slope of CT (moder humus) than at the other sites and was in the order: F > L > A layers. Clamp-bearing hyphal length in Basidiomycota accounted for as much as 19% of the total hyphal length and decreased with the depth of the forest floor and soil in all study sites. Seasonal changes of hyphal length and the layer × month interaction were significant on mull but not on moder humus, suggesting that hyphal length was more sensitive to season in mull than in moder humus.

Keywords Climate · Fungi · Fungal biomass · Mycelia · Seasonal change

Introduction

Fungi are a major component of soil biota in terrestrial ecosystems, representing large pools of nutrients, controlling carbon and nutrient cycling in soils as decomposers and mutualistic root-symbionts, and involved in soil food webs (Dix and Webster 1995; Boddy et al. 2008). The abundance and species composition of fungal communities in litter and soil can be indicative of the functional roles of fungi in soil processes. It is difficult, however, to examine both fungal abundance and species composition concurrently with any single method. Moreover, a number of factors contribute to the complexity in measuring fungal communities in soils. Consequently, a variety of techniques have been described for quantifying fungal biomass in litter and soil (Newell 1992). Commonly used methods to measure fungal biomass include direct microscopic visual observations of hyphal length and biochemical assays for fungal marker molecules, such as chitin and ergosterol. Of these methods, the direct microscopic techniques can be biased by a degree of observer subjectivity, but they allow for visual classification of hyphal types, such as hyaline, darkly pigmented, and clamp-bearing ones, which often can help interpreting the composition of fungal community (Frankland 1982; Kjøller and Struwe 1982; Osono 2007; van der Wal et al. 2009).

Fungal biomass expressed as the hyphal length has been reported using direct observation methods in litter and soil (Kjøller and Struwe 1982; Holden et al. 2013) and in litter at initial stages of decomposition (e.g. Berg and Söderström 1979; Fioretto et al. 1998; Osono and Takeda 2001; Osono 2005). To date, the variations of hyphal length with soil layer, season, humus form, and climatic region have been examined separately. To the knowledge of the author, however, few studies have investigated the effects of soil layer and season on hyphal length at the same time and compared them between sites with different humus forms and climatic conditions (Osono 2011).

The purpose of the present study was to investigate the hyphal length in the forest floor and soil of subtropical, cool temperate, and subalpine forests in Japan with an agar film method. The primary goal was to describe the seasonal variations in hyphal length in different layers of mull and moder humus forms in forests of these three climatic regions. Soil samples were collected at each study forest over a snow-free period to evaluate the relative effects of layer, season, and their interactions on total, darkly pigmented, and clamp-bearing hyphal lengths. Previous data of hyphal lengths in the forest floor and soils of different climatic regions were then summarized to explore whether hyphal length in litter and soil would exhibit patterns along a latitudinal or climatic gradient.

Materials and methods

Study site

Samples were collected from three sites in Japan: a subtropical forest (ST), a cool temperate forest (CT), and a subalpine forest (SA). Table 1 shows the location, climatic condition, vegetation, and data of forest floor measurements of the study sites. ST was located in the northern part of Okinawa Island in southern Japan. A study plot was established in a subtropical broadleaved forest dominated by *Castanopsis sieboldii* (Makino) Hatusima (Fagaceae) and *Schima wallichii* (DC.) Korthals (Theaceae) in the Yona Experimental Forest of the University of the Ryukyus. CT was located in Kyoto, central Japan. Two study plots were established on the lower and upper parts of a northwest-facing slope (approximately 200 m long; Osono et al. 2008a) in a cool temperate forest dominated by *Fagus crenata* Bl. and *Quercus crispula* Bl. (Fagaceae) in the Ashiu

Experimental Forest of Kyoto University. SA was located on Mt. Ontake, Gifu, central Japan. A study plot was established there in a subalpine forest dominated by *Abies mariesii* Masters, *Abies veitchii* Lindley, *Picea jezoensis* var. *hondoensis* (Sieb. et Zucc.) Carrière (Pinaceae), and *Betula ermanii* Cham. (Betulaceae). Monthly temperature data of the sites are shown in Fig. 1. The three sites differed in mean annual temperature and seasonal changes in air temperature, but they received similar amounts of precipitation annually (Table 1, Fig. 1). The mean annual temperature and annual precipitation of the year when the samplings were conducted were 21.1°C and 3167 mm in 2007 in ST, 9.8°C and 2548 mm in 2001 in CT, and 2.0°C and 2299 mm in 2008 in SA. The study sites experience a rainy season from May to June in ST and from June to July in CT and SA. Snow covers the forest floor of CT from December to April and that of SA from mid-November to early June.

Sample collection

A study plot of 50 × 10 m (500 m^2) was laid out in each of ST, CT (upper), CT (lower), and SA sites and was divided into 125 grids of 2 × 2 m. Samples of L, F, and A layer materials were collected from the four plots and used for the estimation of hyphal length. Collection of the samples was performed six times: in March, May, July, September, and November 2007 and in January 2008 in ST, four times: in May, July, September, and November 2001 in CT, and three times: in June, August, and October 2008 in SA. On each sampling occasion, 10 (ST and SA) or five (CT lower and upper) grids were randomly chosen from the 125 grids in each plot, and one soil block (20 × 20 cm in area) was collected from the center of each grid. The block was divided into L and A layers for ST and CT (lower) and L, F, and A layers for CT (upper) and SA, preserved in vinyl bags, and taken to the laboratory. The F layer in ST and CT (lower) developed poorly and was not included in the measurement of hyphal length. The A layer materials (3 cm in depth) were collected from the surface of A layer because the depth of A layer varied between the sites.

Thus, a total of 310 samples were used for the present study, including 120 (6 dates × 2 layers × 10 replicates) from ST, 40 (4 dates × 2 layers × 5 replicates) from CT (lower), 60 (4 dates × 3 layers × 5 replicates) from CT (upper), and 90 (3 dates × 3 layers × 10 replicates) from SA. Samples were preserved in a refrigerator at 4°C and processed within 48 hours after sampling. The L layer materials were fragmented using a blender to make particles of approximately 5 × 5 mm in area, and F and A layer materials were passed through a 2-mm sieve to exclude plant roots and coarse fragments, before a portion of the samples was used for hyphal length estimation.

Hyphal length estimation

Hyphal lengths in L, F, and A layer materials were estimated using the agar film method of Jones and Mollison (1948), but with several modifications (Osono et al. 2006). One gram of a sample was homogenized in a blender at 10,000 rev/min in 49 ml of distilled water for 3 min. The suspension (20 ml) was diluted with 20 ml of molten agar solution (final concentration 1.5%) and mixed at low speed on a magnetic stirring plate. Three agar plates were prepared for each suspension in a haemocytometer (0.1 mm depth), transferred to glass slides, and dried for 24 hours. The films were stained with fluorescent brightener (FB) for one hour. FB binds to chitin in fungal cell walls (West 1988) and enables visualization of all hyaline hyphae that are live or ghosts (empty). The stained films were mounted between slides and coverslips with one drop of immersion oil (type DF, Cargille Laboratories, Inc., Cedar Grove, NJ, USA) and examined with a Nikon Microphot-SA epifluorescent microscope equipped with a high-intensity mercury light source. A Nikon UV-1A filter cube was used for examination of FB-stained hyphae. Darkly pigmented hyphae that were not stained with FB were observed by bright field microscopy. Microscope fields were selected randomly and 25 fields were observed for each slide at 1000× magnification. Hyphal lengths were estimated using an eyepiece grid and a grid-intersection method (Olson 1950). Total hyphal length was calculated as the sum of the lengths of hyaline hyphae stained with FB and darkly pigmented hyphae. Hyphae with clamp connection were classified into Basidiomycota, in spite of the fact that the hyphal length may have been underestimated because the frequency of clamp formation varies between species. Separate litter samples were oven-dried to a constant mass at 40°C and used to measure oven-dry weight.

Statistical analysis

The generalized linear model (GLM) was used to evaluate the difference in hyphal length using layer, month, and the layer × month interaction as independent variables. Tukey's HSD test was performed for multiple comparisons. JMP 6.0 for Macintosh was used to perform these analyses.

Results

Total hyphal length

The mean total hyphal length was generally higher in CT than in ST and SA, and decreased with the depth of the forest floor and soil (Fig. 2). In ST, the mean total hyphal length ranged from 3696 to 6311 m/g in the L layer and from 1061 to 1366 m/g in the A layer (Fig. 2). Total hyphal length in the L layer was significantly higher than that in the A layer (Table 2). The effects of month and layer × month interaction were also significant (Table 2), with total hyphal length in the L layer being especially elevated in November (Fig. 2).

The mean total hyphal length in CT was generally greater at the upper than at the lower CT site (Fig. 2). In the L layer, it ranged from 8259 to 12172 m/g at the lower site and from 12768 to 18733 m/g at the upper site; in the F layer, it ranged from 8243 to 10051 m/g at the upper site; and in the A layer, it ranged from 530 to 2022 m/g at the lower site and from 2795 to 3864 m/g at the upper site. Total hyphal length in the L layer was significantly higher than that in the A layer at both the upper and lower sites (Table 2). Total hyphal length at the lower site also varied significantly with month (Table 2), being higher in May than in June and September (Fig. 2).

The mean total hyphal length in SA ranged from 6061 to 6604 m/g in the L layer, from 3982 to 4769 m/g in the F layer, and from 871 to 1090 m/g in the A layer (Fig. 2). The differences among the layers were statistically significant, whereas significant variation among the months was not observed (Table 2).

Darkly pigmented hyphal length

Overall, darkly pigmented hyphal length was greater at CT (upper) than at the other sites, and decreased in the order: F (if available) > L > A layer (Fig. 3). In ST, the mean darkly pigmented hyphal length ranged from 361 to 859 m/g in the L layer and from 54 to 162 m/g in the A layer (Fig. 3). Darkly pigmented hyphal length was significantly greater in the L than in the A layer, and varied with month (Table 2). It accounted for 5% to 15% of the total hyphal length, and the proportion did not vary significantly with either layer or month (Table 2).

The mean darkly pigmented hyphal length in CT ranged from 942 to 3039 m/g in the L layer at the upper and lower sites, from 2932 to 3212 m/g at the upper site, and from 237 to 827 m/g in the A layer at the upper and lower sites (Fig. 3). Darkly pigmented hyphal length was significantly different in the order: L > A layer at the lower site and F > L > A layer at the upper site, and it did not vary significantly with month at either site (Table 2). The proportion of darkly pigmented hyphal length with respect to the total hyphal length was significantly different between the layers at both the upper and lower sites (Table 2). It was 38% to 53% in the A layer at the lower site and 32% to 37% in the F layer at the upper site, whereas it was between 8% and 26% in the L layer at the lower site and in the L and A layers at the upper site (Fig. 3).

The mean darkly pigmented hyphal length in SA ranged from 806 to 1367 m/g in the L layer, from 1432 to 1799 m/g in the F layer, and from 124 to 425 m/g in the A layer (Fig. 3), and the differences between the layers were statistically significant (Table 2). Darkly pigmented hyphal length accounted for 30% to 45% of the total hyphal length in the F layer, which was significantly higher than that in the L layer (14% to 23%) (Table 2). This proportion was significantly higher in June than in August or October (Fig. 3, Table 2).

Clamp-bearing hyphal length

The mean clamp-bearing hyphal length in ST ranged from 198 to 906 m/g in the L layer and from 2 to 89 m/g in the A layer (Fig. 4), and the difference between the L and A layers was statistically significant (Table 2). The effects of month and the layer × month interaction were also significant (Table 2), indicating that clamp-bearing hyphal length in L layer was significantly elevated, especially in November (Fig. 4). Clamp-bearing hyphal length accounted for 4% to 13% of the total hyphal length in the L layer, and for 0% to 6% in the A layer, and the difference between the L and A layers was statistically significant (Table 2).

The mean clamp-bearing hyphal length at the lower site in CT ranged from 330 to 1049 m/g in the L layer and was not detected in the A layer, whereas at the upper site in CT it ranged from 435 to 3792 m/g in the L layer, 319 to 1130 in the F layer, and 0 to 100 m/g in the A layer (Fig. 4). Clamp-bearing hyphal length and its proportion relative to the total hyphal length was significantly higher in the L than in the F and A layers both at the upper and lower sites and was significantly higher in November than in May at the upper site (Table 2). Clamp-bearing hyphal length generally accounted for 0% to 13% of the total hyphal length and increased to 19% at the upper site in November (Fig. 4).

The mean clamp-bearing hyphal length in SA ranged from 836 to 1012 m/g in the L layer, from 130 to 910 m/g in the F layer, and from 0 to 207 m/g in the A layer (Fig. 4), and the differences between the layers were statistically significant (Table 2). Clamp-bearing hyphal length accounted for 0% to 19% of the total hyphal length (Fig. 4).

Discussion

The values of total hyphal lengths in the present study fell within the range of those previously reported in forests of Japan (Table S1 in Electronic Supplementary Material) and other regions (summarized in Kjøller and Struwe 1982). The mean total hyphal lengths in the L layer of ST, CT, and SA correspond to 9.6, 23.7, and 11.9 mg/g, respectively, calculated with the conversion factors of: the radius of hyphae (r) of 2 μm, the dry matter proportion of hyphae (dm_r) of 0.1, and the density of hyphae (d_f) of 1.5 g/cm^3 (Kjøller and Struwe 1982). These length estimates are again within the range of those previously reported (Kjøller and Struwe 1982). There have been few estimates of the length of darkly-pigmented or clamp-bearing hyphae, but the values in the present study were consistent with those previously reported in Japan (Table S1). Similarly, Bååth and Söderström (1977) reported that the length of darkly pigmented and clamp-bearing hyphae in a Swedish coniferous forest ranged from 100 to 3500 m/g and from 20 to 1300 m/g, respectively, and accounted for 14% to 27% and 2% to 13% of the total hyphal length, respectively. The results of the present study (Fig. 2) are consistent with the statement of Frankland (1982) that fungal mycelia are less concentrated in mull with a thin accumulation of litter than in mor and moder with well-developed organic layers.

The lengths of total and clamp-bearing hyphae decreased with the depth of soil horizons in the present study (Figs. 2 and 4), which is in agreement with previous reports (Ruscoe 1971; Hunt and Fogel 1983). These vertical patterns are primarily attributable to the decrease in carbon content with depth (Kjøller and Struwe 1982). In contrast, darkly pigmented hyphal length increased in F layers of moder humus [i.e. in CT (upper) and SA] (Fig. 3). Bååth and Söderström (1977) and Osono et al. (2003) also reported higher values of darkly pigmented hyphal length in deeper soil layers. Decomposition experiments have also demonstrated an increase in the length and/or the proportion of darkly pigmented hyphae during litter transformation (Osono 2005; Osono et al. 2014). Osono et al. (2006) found that incubation of needle litter beneath the L layer resulted in a significantly greater amount of darkly pigmented hyphae than incubation of the same litter on the surface of the L layer. Suggested underlying mechanisms of an increase of darkly pigmented hyphae thus should include the higher potential of dematiaceous fungi to colonize litter in deeper layers (e.g. Tokumasu 1998) and the slow turnover of recalcitrant melanized hyphae (Butler and Day 1998). Relatively high values of darkly pigmented hyphal length and its proportion (29% to 42%) relative to the total length were found in needle litter of *Chamaecyparis obtusa* and in arctic substrates (Table S1). This suggests that darkly pigmented hyphae can increase under stress of nutrients (e.g. in needle litter) or environmental conditions (e.g. low temperatures or freezing), possibly in association with the stress-resistant nature of melanized hyphae (Butler and Day 1998).

The total hyphal length in the L layer increased significantly in November in ST and in May in CT (lower) (Fig. 2), where mull humus developed (Table 1). Previous studies already showed an increase of total hyphal length in spring and autumn and attributed it to favorable climatic (moisture and temperature) and nutrient factors for fungal growth (Ruscoe 1971; Bååth and Söderström 1977). A marked increase in clamp-bearing hyphal length and its proportion relative to the total length in CT (upper) (Fig. 4) may be due to an increase in litter-decomposing and/or mycorrhizal basidiomycetes. A concomitant increase in the occurrence of fruiting bodies of litter-decomposing and mycorrhizal basidiomycetes was also observed in that November in CT (Osono unpubl.). Ruscoe (1971) also reported an autumn increase in clamp-bearing hyphal length in soil of a pure stand of *Nothofagus truncata*, but the generality and ecological significance of the autumn flush of basidiomycetes remain unclear, mainly due to the lack of relevant studies. Limitations of the present study should also be noted as the field surveys were performed for different single years at the three sites and only for snow-free periods at CT and SA.

Generally, the effects of layer of the forest floor were more marked on the total, darkly pigmented, and clamp-bearing hyphal lengths than the month of sampling at study sites (Table 2). Interestingly, the seasonal changes of total hyphal length were significant on mull humus at ST and CT (lower) but not on moder humus at CT (upper) and SA (Table 2). The effect of layer × month interaction on total, darkly pigmented, and clamp-bearing hyphal length was also significant only at ST and CT (lower). These results suggest that hyphal length in L layer was more sensitive to season in mull than in moder soils. Possible explanations for this sensitivity are the amount and the turnover of forest floor materials. The lower amount and the faster turnover of the forest floor in mull soils (Tsukamoto 1996) may lead to more variable hyphae length than in moder humus.

When data of hyphal length in the forest floor and soils of Asian forests and Canadian arctic tundra examined with the same direct observation method were plotted against mean annual temperatures (Table S1), peaks of the total, darkly pigmented, and clamp-bearing hyphal length in temperate regions would be implied (Fig. S1). It is difficult, however, to gain insights into climatic patterns of hyphal length, as data are still lacking in soils of other than temperate regions. For example, Widden and Parkinson (1979) reported that total hyphal length in Canadian tundra soils reached 2228 m/g, whereas Robinson et al. (1996) summarized the data of total hyphal length in soils and dead plant tissues on tundra soils and reported that these ranged from 4 to 9600 m/g dry soil. Further efforts are needed to measure hyphal lengths in various soils, especially of tropical regions.

Acknowledgments I thank Dr. A. Takashima and staff at the Yona

Experimental Forest, University of the Ryukyus, for help with fieldwork at ST; the staff at Ashiu Experimental Forest, Kyoto University, for help with fieldwork at CT; and Dr. Elizabeth Nakajima for critical reading of the manuscript. This study received partial financial support from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT) (No. 19780114), The Sumitomo Foundation, Nissan Global Foundation, Nippon Life Inst. Foundation, and Grants for Excellent Graduate Schools, MEXT, Japan (12-01) to Kyoto University.

References

Bååth E, Söderström BE (1977) Mycelial lengths and fungal biomasses in some Swedish coniferous forest soils, with special reference to a pine forest in central Sweden. Swedish Coniferous Forest Project, Technical Report 13:1-45

Berg B, Söderström B (1979) Fungal biomass and nitrogen in decomposing Scots pine needle litter. Soil Biol Biochem 11:339-341

Boddy L, Frankland JC, van West P (2008) Ecology of Saprotrophic

Basidiomycetes. Academic Press, London

Butler MJ, Day AW (1998) Fungal melanins: a review. Can J Microbiol 44:1115-1136

Dix NJ, Webster J (1995) Fungal Ecology. Chapman & Hall, London

Enoki T (2003) Microtopography and distribution of canopy trees in a subtropical evergreen broad-leaved forest in the northern part of Okinawa Island, Japan. Ecol Res 18:103-113

Fioretto A, Musacchio A, Andolft G, Virzo de Santo A (1998) Decomposition dynamics of litters of various pine species in a Corsican pine forest. Soil Biol Biochem 30:721-757

Frankland JC (1982) Biomass and nutrient cycling by decomposer basidiomycetes. In: Frankland JC, Hedger JN, Swift Mj (eds) Decomposer Basidiomycetes, Their Biology and Ecology. Cambridge University Press, Cambridge, pp 241-261

Hobara S, Osono T, Hirose D, Noro K, Hirota M, Benner R (2014) The roles of microorganisms in litter decomposition and soil formation. Biogeochemistry 118:471-486

Holden SR, Gutierrez A, Treseder KK (2013) Soil fungal communities, extracellular enzyme activities, and litter decomposition across a fire chronosequence in Alaskan boreal forests. Ecosystems 16:34-46

Hunt GA, Fogel R (1983) Fungal hyphal dynamics in a western Oregon Douglas-fir stand. Soil Biol Biochem 15:641-649

Jones PCT, Mollison JE (1948) A technique for the quantitative estimation of soil microorganisms. J Gen Microbiol 2:54-69

Kjøller A, Struwe S (1982) Microfungi in ecosystems: fungal occurrence and activity in litter and soil. Oikos 39:391-422

Koide K, Osono T, Takeda H (2005) Fungal succession and decomposition of *Camellia japonica* leaf litter. Ecol Res 20:599-609

Mori A, Mizumachi E, Osono T, Doi Y (2004) substrate-associated seedling recruitment and establishment of major conifer species in an old-growth subalpine forest in central Japan. For Ecol. Manag 196:287-297

Newell SY (1992) Estimating fungal biomass and productivity in decomposing litter. In: Carroll GC, Wicklow DT (eds) The Fungal Community: Its Organization and Role in the Ecosystem. Marcel Dekker, New York, pp

521-561

Olson FCW (1950) Quantitative estimates of filamentous algae. T Am Microsc Soc 69:272-279

Osono T (2005) Colonization and succession of fungi during decomposition of *Swida controversa* leaf litter. Mycologia 97:589-597

Osono T (2007) Ecology of ligninolytic fungi associated with leaf litter decomposition. Ecol Res 22:955-974

Osono T (2011) Diversity and functioning of fungi associated with leaf litter decomposition in Asian forests of different climatic regions. Fun Ecol 4:375-385

Osono T, Takeda H, (2001) Organic chemical and nutrient dynamics in decomposing beech leaf litter in relation to fungal ingrowth and succession during three year decomposition processes in a cool temperate deciduous forest in Japan. Ecol Res 16:649-670

Osono T, Hobara S, Fujiwara S, Koba K, Kameda K (2002) Abundance, diversity, and species composition of fungal communities in a temperate forest affected by excreta of the Great Cormorant *Phalacrocorax carbo*. Soil Biol

Biochem 34:1537-1547

Osono T, Ono Y, Takeda H (2003) Fungal ingrowth on forest floor and decomposing needle litter of *Chamaecyparis obtusa* in relation to resource availability and moisture condition. Soil Biol Biochem 35:1423-1431

Osono T, Hirose D, Fujimaki R (2006) Fungal colonization as affected by litter depth and decomposition stage of needle litter. Soil Biol Biochem 38:2743-2752

Osono T, Takeda H (2007) Microfungi associated with *Abies* needles and *Betula* leaf litter in a subalpine coniferous forest. Can J Microbiol 53:1-7

Osono T, Takeda H, Azuma JI (2008a) Carbon isotope dynamics during leaf litter decomposition in a cool temperate forest with reference to lignin fractions. Ecol Res 23:51-55

Osono T, Ishii Y, Takeda H (2008b) Fungal colonization and decomposition of *Castanopsis sieboldii* leaves in a subtropical forest. Ecol Res 23:909-917

Osono T, Ishii Y, Takeda H, Seramethakun T, Khamyong S, To-Anun C, Hirose D, Tokumasu S, Kakishima M (2009) Fungal succession and lignin decomposition on *Shorea obtusa* leaves in a tropical seasonal forest in northern Thailand. Fungal Diversity 36:101-119

Osono T, Hobara S, Hishinuma T, Azuma JI (2011) Selective lignin decomposition and nitrogen mineralization in forest litter colonized by *Clitocybe* sp. Eur J Soil Biol 47:114-121

Osono T, Ueno T, Uchida M, Kanda H (2012) Abundance and diversity of fungi in relation to chemical changes in arctic moss profiles. Polar Sci 6:121-131

Osono T, Matsuoka S, Hirose D, Uchida M, Kanda H (2014) Fungal colonization and decomposition of leaves and stems of *Salix arctica* on deglaciated moraines in high-Arctic Canada. Polar Sci 8:in press

Robinson CH, Borisova OB, Callaghan TV, Lee JA (1996) Fungal hyphal length in litter of Dryas octopetala in a high-Arctic polar semi-desert, Svalbard. Polar Biol 16:71-74

Ruscoe QW (1971) The soil mycoflora of a hard beech forest. New Zealand Journal of Science 14:554-567

Takeda H, Kaneko N (1988) Patterns of soil humus accumulation in forests. I. Mull and moder types humus in a broad-leaved forest. Bull Kyoto Univ

Forest 60:33-45 (in Japanese with English abstract)

Tateno R, Takeda H (2003) Forest structure and tree species distribution in relation to topography-mediated heterogeneity of soil nitrogen and light at the forest floor. Ecol Res 18:559-571

Tian X, Takeda H, Ando T (1997) Application of a rapid thin section method for observations on decomposing litter in mor humus form in a subalpine coniferous forest. Ecol Res 12:289-300

Tokumasu S (1998) Fungal successions on pine needles fallen at different seasons: the succession of surface colonizers. Mycoscience 39:417-423

Tsukamoto J (1996) Soil macro-invertebrates and litter disappearance in a Japanese mixed deciduous forest and comparison with European deciduous forests and tropical rainforests. Ecol Res 11:35-50

van der Wal A, Bloem J, Mulder C, de Boer W (2009) Relative abundance and activity of melanized hyphae in different soil ecosystems. Soil Biol Biochem 41:417-419

West AW (1988) Specimen preparation, stain type, and extraction and observation procedures as factors in the estimation of soil mycelial lengths and volumes by light microscopy. Biol Fertil Soils 7:88-94

Widden P, Parkinson D (1979) Populations of fungi in a high arctic ecosystem. Can J Bot 57:2408-2417

Figure legends.

Fig. 1. Seasonal changes in air temperature at the study sites. □, subtropical (ST, Okinawa); ●, cool temperate (CT, Kyoto); ▲, subalpine forest (SA, Gifu). Values are long-term averages for 22 to 35 years.

Fig. 2. Total hyphal length in soil layers. Values are mean ± standard errors. □, L layer; ●, F layer; ▲, A layer. Note that the scales of the Y-axis are not consistent between the sites.

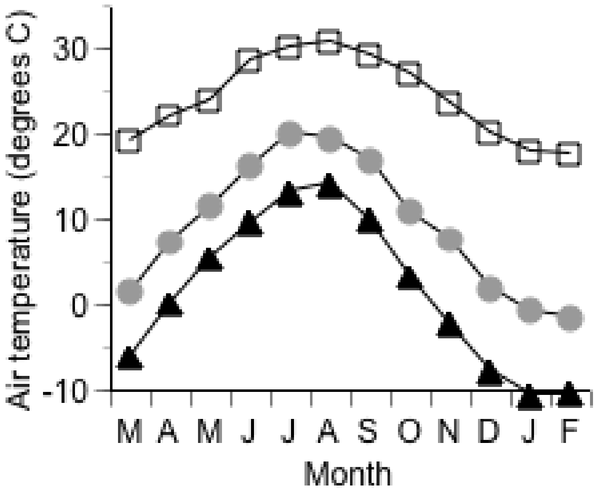
Fig. 3. Darkly pigmented hyphal length (upper) and its proportion relative to the total hyphal length (lower) in soil layers. Values are mean ± standard errors. Symbols are as in Fig. 2. Note that the scales of the Y-axis are not consistent between the sites.

Fig. 4. Clamp-bearing hyphal length (upper) and its proportion relative to the

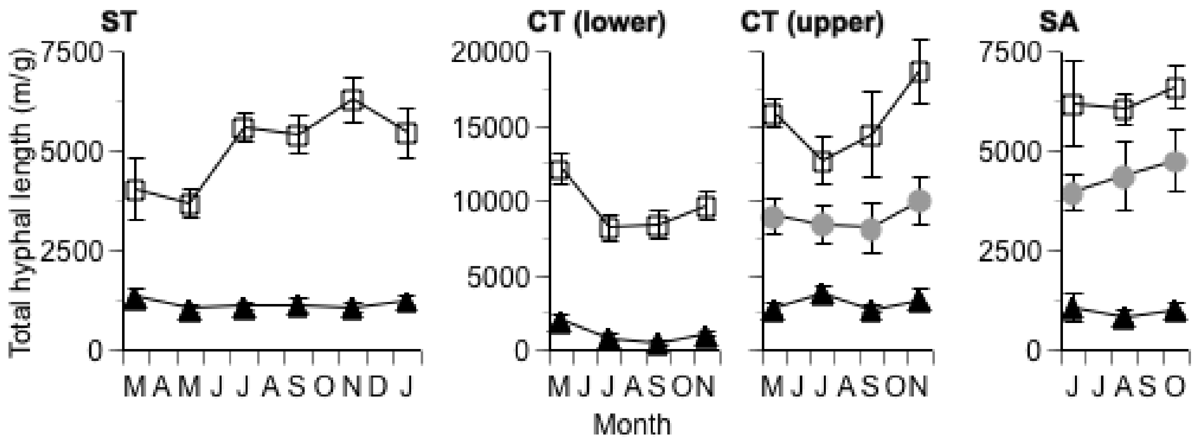
total hyphal length (lower) in soil layers. Values are mean ± standard errors. Symbols are as in Fig. 2. Note that the scales of the Y-axis are not consistent between the sites.

1 Osono Fig. 1

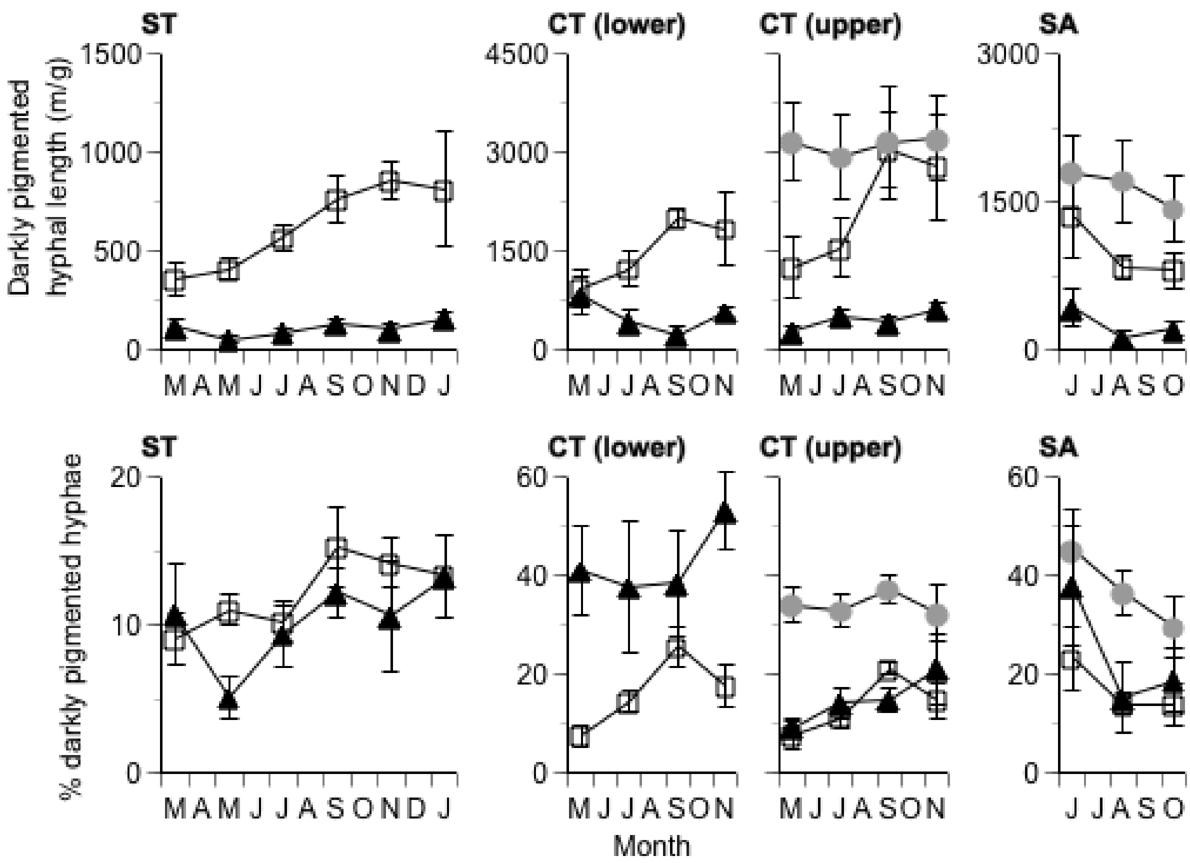
2



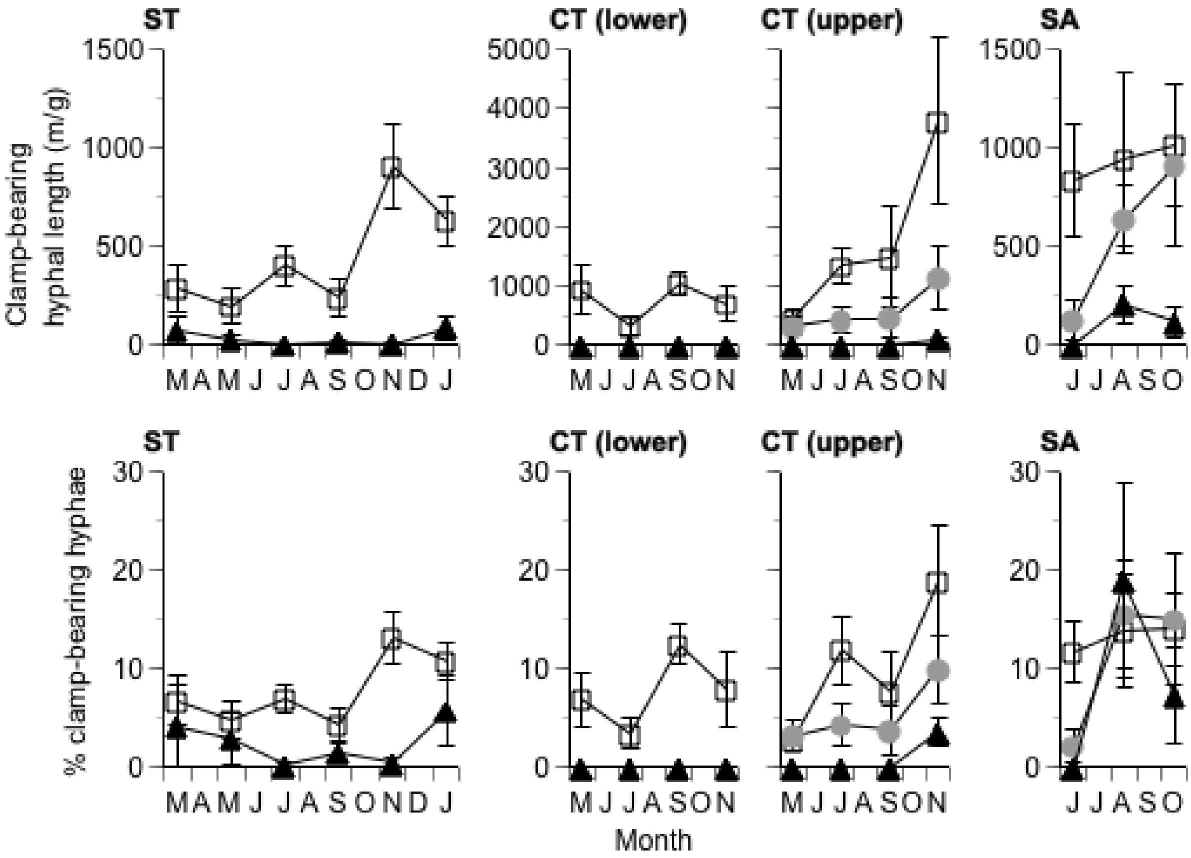
Osono Fig. 2


ST
CT (lower)
CT (upper)
SA
Total hyphal length (m/g)
7500
5000
2500
0
20000
15000
10000
5000
0
7500
5000
2500
0
M A M J J A S O N D J
M J J A S O N
M J J A S O N
J J A S O
Month

Osono Fig. 3


ST
CT (lower)
CT (upper)
SA
Darkly pigmented hyphal length (m/g)
0
500
1000
1500
1500
3000
4500
3000
M A M J J A S O N D J
M J J A S O N
J J A S O
% darkly pigmented hyphae
0
10
20
20
40
60
Month

1 Osono Fig. 4


ST
CT (lower)
CT (upper)
SA
Clamp-bearing hyphal length (m/g)
% clamp-bearing hyphae
Month
M A M J J A S O N D J
M J J A S O N
J J A S O
0
500
1000
1500
2000
3000
4000
5000
10
20
30

Osono Table 1

Table 1. Location, climate, vegetation, and properties of the forest floor in the study sites.

Site ID	ST	CT	SA
Location			
Latitude N	26°49'	35°18'	35°56'
Longitude E	128°50'	135°43'	137°28'
Elevation (m)	330	660	2050
Site	Kunigami, Okinawa	Ashiu, Kyoto	Mt. Ontake, Gifu
Climate			
Mean annual temperature (°C)	22[a]	10[b]	2[c]
Annual precipitation (mm)	2456[a]	2495[b]	2500[c]
Climate region	Subtropical	Cool temperate	Subalpine
Vegetation			
Type	Evergreen broad-leaved	Deciduous broad-leaved	Evergreen coniferous
Dominant tree species	*Castanopsis sieboldii, Schima wallichii*[d]	*Fagus crenata, Quercus crispula*[e]	*Abies mariesii, A. veitchii, Picea jezoensis* var. *hondoensis, Betula ermanii*[f]
Forest floor			
Humus type	Mull	Mull (lower), Moder (upper)[g]	Moder[h]
Depth of L layer (cm)[i]	1.1 ± 0.1 b	1.2 ± 0.1 b	2.7 ± 0.3 a
Depth of F layer (cm)[i]	1.0 ± 0.2 c	4.0 ± 0.4 b	13.4 ± 1.2 a

[a]Osono et al. (2008b), [b]Ashiu Experimental Forest, Kyoto University, [c]Osono and Takeda (2007), [d]Enoki (2003), [e]Tateno and Takeda (2003), [f]Mori et al. (2004), [g]Takeda and Kaneko (1988), [h]Tian et al. (1997).

[i]Values indicate means ± standard errors (n=20) for the depths of L and F layers. Measurement was carried out in the three study sites in October 2012. Values of CT were from the lower slope. The same letters indicate that the values were not statistically different at 5% level with Tukey's HSD test.

Osono Table 2

Table 2. Summary of generalized linear models for hyphal length. χ^2 values are indicated. *** P<0.001, ** P<0.01, * P<0.05, ns not significant.

	Model		Layer		Month		Layer × Month	
Total hyphal length								
ST	165.0	***	155.7	***	15.7	**	17.5	**
CT (lower)	88.3	***	84.9	***	16.7	***	4.9	ns
CT (upper)	82.3	***	78.9	***	5.6	ns	6.1	ns
SA	79.4	***	78.8	***	0.8	ns	0.6	ns
Darkly pigmented hyphal length								
ST	79.6	***	65.7	***	14.4	*	9.8	ns
CT (lower)	28.4	***	20.5	***	2.6	ns	9.6	*
CT (upper)	45.9	***	40.4	***	4.3	ns	5.7	ns
SA	37.1	***	34.0	***	3.4	ns	1.1	ns
% darkly pigmented hyphae								
ST	15.3	ns	2.3	ns	9.9	ns	3.7	ns
CT (lower)	23.3	**	20.9	***	3.0	ns	3.1	ns
CT (upper)	68.6	***	61.9	***	9.4	*	7.8	ns
SA	22.5	**	13.4	**	8.8	*	1.8	ns
Clamp-bearing hyphal length								
ST	75.8	***	48.6	***	20.5	**	21.4	***
CT (lower)	26.6	***	21.7	***	4.0	ns	4.0	ns
CT (upper)	38.8	***	21.7	***	12.6	**	11.9	ns
SA	20.2	**	15.4	***	3.4	ns	2.3	ns
% Clamp-bearing hyphae								
ST	31.0	**	16.5	***	8.7	ns	8.4	ns
CT (lower)	32.2	***	25.3	***	6.3	ns	6.3	ns
CT (upper)	41.7	***	23.7	***	18.2	***	7.8	ns
SA	12.2	ns	1.2	ns	7.7	*	3.8	ns

Electronic Supplementary Material

Hyphal length in the forest floor and soil of subtropical, temperate, and subalpine forests

Takashi Osono

S1. A review of hyphal length in forest and tundra litter examined with the same direct observation method. MAT, mean annual temperature; AP, annual precipitation. na, not available. Numbers in parentheses are the proportions relative to the total hyphal length.

Location	Climate	MAT °C	AP mm	Vegetation	Tree species	Sample	Total hyphal length m/g	Darkly pigmented hyphal length m/g	Clamp-bearing hyphal length m/g	Ref.
Kyoto, Japan	Cool temperate	10	2495	Deciduous broad-leaved	*Fagus crenata*	Litter	7867	983 (12)	410 (5)	1
Shiga, Japan	Warm temperate	15	1475	Cypress plantation	*Chamaecyparis obtusa*	L layer	12334	5800 (42)	1191 (10)	2
Kyoto, Japan	Warm temperate	15	1734	Secondary	*Chamaecyparis obtusa*	Litter	7595	970 (13)	79 (1)	3
						L2 layer	12523	2943 (24)	560 (4)	
						F layer	9549	2805 (29)	119 (1)	
						H layer	6932	2546 (37)	167 (2)	
Kyoto, Japan	Cool temperate	10	2495	Deciduous broad-leaved	*Swida controversa*	Litter	5309	925 (16)	111 (2)	4
Kyoto, Japan	Warm temperate	15	1581	Secondary	*Camellia japonica*	Litter	7824	998 (13)	136 (2)	5

Kyoto, Japan	Warm temperate	15	1734	Secondary	*Chamaecyparis obtusa*	Litter	12321	3622 (29)	154 (1)	6
				Pine plantation	*Pinus pentaphylla*	Litter	10385	1337 (13)	336 (3)	
Okinawa, Japan	Subtropical	22	2456	Evergreen broad-leaved	*Castanopsis sieboldii*	Litter	6593	525 (9)	556 (8)	7
Chiang Rai, Thailand	Tropical seasonal	25	1155	Dry dipterocarpus	*Shorea obtusa*	Litter	2738	551 (19)	80 (2)	8
Kyoto, Japan	Cool temperate	10	2495	Deciduous broad-leaved	*Fagus crenata*	L layer	6927	1001 (13)	na	9
Nunavut, Canada	Arctic	-20	64	Tundra	*Hylocomium splendens*	Moss	4446	1859 (41)	658 (13)	10
					Racomitrium lanuginosum	Moss	1164	349 (30)	107 (5)	
Nunavut, Canada	Arctic	-20	64	Tundra	*Salix arctica*	Litter	4068	1063 (30)	145 (2)	11
Nagano, Japan	Cool temperate			Grassland	*Miscanthus sinensis*	Litter	4720	395 (10)	155 (3)	12
				Secondary coniferous	*Pinus densiflora*	Litter	10273	1933 (20)	1454 (14)	
				Deciduous broad-leaved	*Quercus crispula*	Litter	3775	490 (15)	320 (9)	
Okinawa, Japan	Subtropical	22	2456	Evergreen broad-leaved	*Castanopsis sieboldii*	L layer	5092	630 (12)	444 (8)	13
						A layer	1175	110 (10)	36 (3)	
Kyoto, Japan	Cool temperate	10	2495	Deciduous broad-leaved	*Fagus crenata* (mull)	L layer	8514	1603 (22)	627 (7)	
						A layer	1139	510 (41)	0 (0)	
					Fagus crenata (moder)	L layer	15473	2154 (14)	1761 (10)	
						F layer	8949	3118 (34)	585 (5)	
						A layer	3242	451 (15)	22 (1)	
Gifu, Japan	Subalpine	2	2500	Evergreen coniferous	*Abies* spp., *Betula ermanii*	L layer	6289	1004 (17)	929 (13)	
						F layer	4382	1649 (37)	559 (11)	
						A layer	998	255 (24)	109 (9)	

Reference. 1, Osono and Takeda (2001); 2, Data of control site, Osono et al. (2002); 3, Osono et al. (2003); 4, means for sterilized and unsterilized litter, Osono (2005); 5, means for bleached and nonbleached portions, Koide et al. (2005); 6, means for four treatments, Osono et al. (2006); 7, means for bleached and nonbleached portions, Osono et al. (2008b); 8, Osono et al. (2009); 9, data of nonbleached litter, Osono et al. (2011); 10, means for horizontal layers, Osono et al. (2012); 11, Osono et al. (2014); 12, Hobara et al. (2014); 13, the present study.

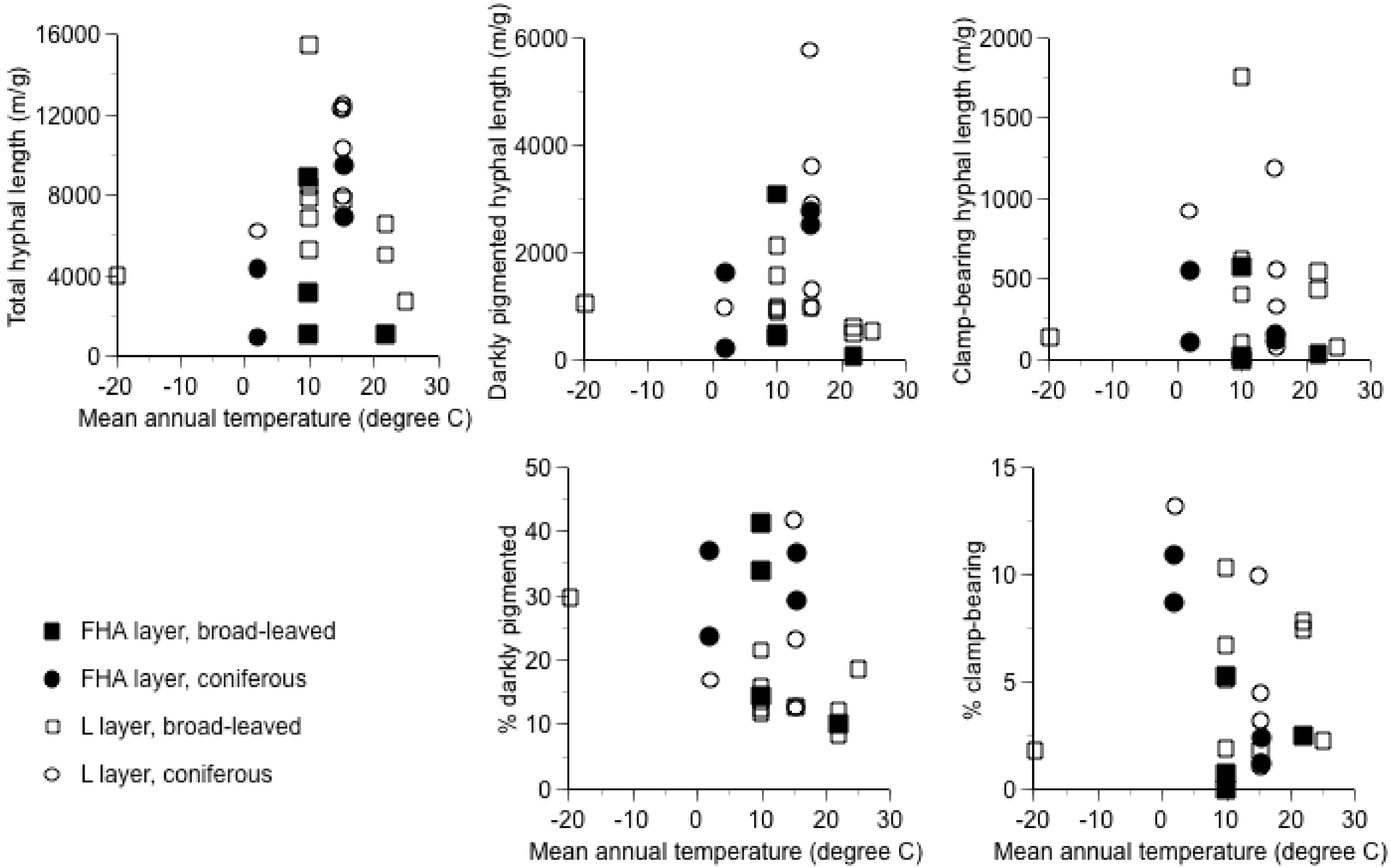


Fig. S1. Hyphal length plotted against mean annual temperature. Data are after Table S1. Data of arctic moss are not included.